# *Clostridium Perfringens* Growth from Spore Inocula in *Sous-Vide* Processed Pork-Based Mexican Entrée

DENISE Y. MIGUEL-GARCIA, VIJAY K. JUNEJA, MARTIN VALENZUELA-MELENDREZ, MARTHA E. DÍAZ-CINCO, H. THIPPAREDDI, AND E. AIDA PEÑA-RAMOS

**ABSTRACT: The combined effect of Citricidal® wih irradiation on *Clostridium perfringens* growth from spores in a *sous-vide* processed marinated pork meat Mexican entrée was investigated. Citricidal was added at 200 or 800 ppm after mixing pork meat with tomatillo sauce and inoculated with 3 $\log_{10}$ CFU/g of *C. perfringens* spores. Samples were irradiated at either 0 or 2 kGy, heated to an internal temperature of 71 °C, and stored at 4 °C for 28 d, 15 °C for 45 d, and 25 °C for 26 h. To simulate the conditions that may occur during transportation, distribution, storage, or handling in supermarkets or by consumers, the effect of static temperature abuse on *C. perfringens* growth was assessed by transferring samples stored at 4 to 25 °C for 13 and 15 h. Total *C. perfringens* populations were determined by plating diluted samples on tryptose-sulfite-cycloserine agar. Growth was not observed up to 45 d of storage at 15 °C in samples supplemented with 800 ppm of Citricidal. At 25 °C, no significant differences ($P > 0.05$) on the lag phase duration due to antimicrobial treatments was observed. The temperature abuse of refrigerated products for up to 15 h did not lead to *C. perfringens* growth to high infective dose levels of 1 million cells required to cause food poisoning. The results suggest that 800 ppm Citricidal can have significant bacteriostatic activity against *C. perfringens* and may provide a degree of protection against this pathogen in *sous-vide* processed marinated pork meat Mexican entrée, under mild temperature abuse (≤ 15 °C) conditions.**

**Keywords: Citricidal, *Clostridium perfringens*, gamma irradiation, pork meat, *sous-vide***



## Introduction

The demand for fresh tasting, preservative free, ready-to-eat foods has led to the development of minimally processed foods, such as *sous-vide* products. This method is defined as fresh food cooked under vacuum and under controlled conditions of time–temperature (Schellekens 1996). The thermal process is not designed to result in sterile products, and thus, may allow the survival of heat resistant spores of pathogens such as *Clostridium perfringens*. Therefore, it is necessary to assure a good cold chain during distribution. However, there is evidence that temperature abuse can occur at some point during the distribution of refrigerated products, thereby increasing the risk of growth of the surviving spore forming pathogens (NFPA 1998).

Vegetative cells of *C. perfringens* are destroyed by heat during the production of ready-to-eat foods, while spores can survive and could be activated by heat during the cooking processes. If these foods are not properly cooled and stored under appropriate refrigeration, heat-activated spores can germinate. Because refrigeration alone may not assure safety of *sous-vide* products, additional safety barriers should be incorporated in such foods.

The hurdle technology is based on the application of combined preservative factors to achieve stable and microbiologically safe food products. A synergistic effect could be achieved if the hurdles act on different targets affecting the homeostasis of the microorganisms (Leistner 2000). Gombas and Gomez (1978) reported that gamma irradiation had a sensitization effect on the subsequent heat resistance of *C. perfringens* spores, but heat treatment had no effect on subsequent radiation resistance.

It has been reported that citrus-related products have antimicrobial activity (Subba and others 1967). Citricidal® is a commercially available antimicrobial made from grapefruit seeds and pulp (Nutriteam Inc., Ripton, Vt., U.S.A.) and the suggested components contributing to the antimicrobial activity are ascorbic acid, citric acid, and flavonoids (Cvetnic and Vladimir 2005). Juneja and others (2006b) reported inhibition of *C. perfringens* germination and outgrowth in marinated *sous-vide* chicken products by the addition of 200 ppm of Citricidal, during storage at 19 and 25 °C. The purpose of this study was to evaluate the combined effect of low-dose irradiation and grapefruit extract on the fate of *C. perfringens* spores during storage of *sous-vide* processed pork-based Mexican entree.

## Materials and Methods

### Test organisms and spore production

Three strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13), from the American Type Culture Collection (ATCC; Manassas, Va., U.S.A.), were obtained from the Microbial Food Safety Research Unit culture collection (Wyndmoor, Pa., U.S.A.). The sporulation process was carried out in Duncan and Strong sporulation medium as previously described (Juneja and others 1993). Spore crops of each strain were then washed twice with sterile distilled water and

*MS 20080877 Submitted 11/4/2008, Accepted 2/14/2009.* *Authors Miguel-Garcia, Valenzuela-Melendrez, Díaz-Cinco, and Aida Peña-Ramos are with Centro de Investigación en Alimentación y Desarrollo, A.C., Carretera a la Victoria Km. 0.6 Hermosillo, Sonora, México CP 83000. Author Juneja is with Microbial Food Safety Research Unit, USDA/ARS Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA 19038, U.S.A. Author Thippareddi is with Dept. of Food Science and Technology, Univ. of Nebraska, Lincoln, NE 68583, U.S.A. Direct inquiries to author Aida Peña-Ramos (E-mail: aida@ciad.mx).*




doi: 10.1111/j.1750-3841.2009.01131.x


THE Society for Food Science & Technology
Journal of Food Science
A Publication of the Institute of Food Technologists

then resuspended in sterile distilled water. The suspensions were stored in a refrigerator at 4 °C. Spore population was heat-shocked for 20 min at 75 °C and enumerated by spiral plating (Model D, Spiral Biotech, Bethesda, Md., U.S.A.) of appropriate dilutions (in 0.1% peptone water), in duplicate, on tryptose-sulfite-cycloserine (TSC) agar followed by incubation of plates anaerobically for 48 h at 35 °C. Immediately before the experiment, an equivalent proportion of each strain suspension was mixed together to prepare a 3-strain cocktail inoculums at a final target level of 2 log spores per gram.

## Sample preparation and inoculation

Sliced pork shoulders (10 × 5 × 1 cm) were obtained from a local retail market (Wyndmoor, Pa., U.S.A.) and precooked at 90 °C for 1 h in a water bath (Thermo Neslab, Digital Plus, Newington, N.H., U.S.A.) and then stored frozen (−20 °C) until used. To get 800 ppm of Citricidal (Nutriteam Inc.) liquid concentrate (60% grapefruit extract), 1.6 g of this compound was added to 500 g of tomatillo sauce (Salsa Verde Herdez®, Melting Pot Foods, Oak Park, Ill., U.S.A.). Likewise, 0.42 g of Citricidal were added to 500 g of Salsa Verde Herdez to get 200 ppm. The use of 500 ppm in preliminary studies gave same results as 200 ppm. Therefore, 200 and 800 ppm were used in the present study. Both treatments were supplemented with 1% of sodium chloride. Thereafter, 2 g of Salsa Verde Herdez of each treatment were weighed in a stomacher bag (Whirl-Pak® long-term sample retention bags, Nasco, Fort Atkinson, Wis., U.S.A.), and then 3 g of minced meat (thawed overnight at room temperature) were added and mixed using a stomacher for 2 min. Both Salsa Verde Herdez and pork meat were tested to be free of *C. perfringens* population. Each bag was inoculated with *C. perfringens* spore cocktail to obtain a final concentration of approximately 3 $\log_{10}$ CFU/g. Noninoculated samples were also prepared. Finally, these bags were evacuated to a negative pressure of 1000 mBar and heat sealed using a Multivac Model A300/16 gas packaging machine (Sepp Haggemuller GmbH & Co., Wolfertschwenden, Germany).

## Thermal process, storage, and temperature abuse

Samples were placed in a basket and submerged in a temperature controlled water bath (Thermo Neslab, Digital Plus) stabilized at 25 °C and increased linearly in a period of 1 h to a final cooking temperature of 71 °C for 2 min; cooled for 5 min in ice water bath at 4 °C. Vacuum-packed meat samples were stored at 4, 15, or 25 °C. Samples stored at 25 °C were analyzed at 0, 3, 6, 9, 13, 16, 18, 20, 22, 24, and 26 h; those at 15 °C were analyzed at 0, 8, 15, 22, 30, and 45 d; and the samples stored at 4 °C were analyzed on 0, 7, 14, 21, and 28 d. *C. perfringens* populations were enumerated on scheduled sampling days and noninoculated samples were used to determine pH and water activity (Aw) of the product using pH meter AB 15 (Fischer Scientific, Singapore) and an Aqua lab model CX-2 (Decagon Services Inc., Pullman, Wash., U.S.A.) water activity meter, respectively. To determine the effect of static temperature abuse, samples stored at 4 °C were moved on their scheduled sampling day (days 7, 14, 21, and 28) to 25 °C and held at this temperature for 13 and 15 h, then plated as described subsequently.

## Irradiation process

Half ot the samples prepared at the conditions previously described, were submitted to irradiation on a gamma irradiation unit of $Ce^{137}$ at the irradiation facility of Eastern Regional Research Center (Wyndmoor, Pa., U.S.A.) at a dose rate of 0.087 kGy/min for a final dose of 2 kGy, at 4 °C. Then, irradiated samples were cooked, stored, and tested as described previously.

## Bacterial enumeration

Total *C. perfringens* population was enumerated by spiral plating (Spiral Plate 4000, Spiral Biotech, Gaithersburg, Md., U.S.A.) on TSC agar as described previously (Juneja and others 1994). Counts were determined after 48 h of anaerobic incubation at 37 °C. Two independent replications of the study were performed with duplicate samples for each sampling period.

## Data processing and experimental design

Bacterial growth curves were generated from the experimental data by Baranyi model (Baranyi and Roberts 1994) using DMFit, a nonlinear regression Excel add-in. The effect of antimicrobial treatments on growth parameters (lag phase and growth rate), were determined by analysis of variance (ANOVA) using NCSS 2000 statistical software (Kaysville, Utah, U.S.A.). Duncan mean separation test was used to determine significant differences ($P < 0.05$) among means. A 2 × 3 factorial arrangement in a block design with 2 replicates was employed, with Citricidal levels (0, 200, and 800 ppm) and irradiation dose (0 and 2 kGy) as main effects, and storage temperature as block factor (4, 15 and 25 °C).

# Results and Discussion

Growth of *C. perfringens* from a spore inoculum was not observed in marinated pork samples cooked to 71 °C and stored at 4 °C in both irradiated and nonirradiated samples with or without Citricidal (data not shown). Similar observations were reported in previous studies (Juneja and Majka 1995) for *C. perfringens* growth in ground beef.

*C. perfringens* growth curves and Baranyi model regressions in marinated pork meat with (1) and without (2) gamma irradiation and different Citricidal levels, subsequently stored at 15 °C are shown in Figure 1. In both irradiated and nonirradiated samples, growth was not observed up to 45 d of storage at 15 °C in samples supplemented with 800 ppm of Citricidal. However, *C. perfringens* population levels at 45 d increased 2 $\log_{10}$ CFU/g in both irradiated and nonirradiated samples with or without 200 ppm of Citricidal. The application of ionizing radiation prior to cooking did not have any effect on the fate of *C. perfringens* in marinated pork.

While published literature on the growth of this pathogen at 15 °C in pork meat is not available, growth in other meat species, such as chicken and beef have been assessed. Juneja and others (2006b) reported growth of *C. perfringens* in marinated *sous-vide* chicken supplemented with 200 ppm of Citricidal increased to 7 $\log_{10}$ CFU/g after 10 d of storage at 19 °C. Similarly, growth of *C. perfringens* vegetative cells (up to 8 $\log_{10}$ CFU/g) was observed during storage of vacuum packaged cooked beef at 15 °C after 40 d (Juneja and others 1994). Comparatively slower *C. perfringens* growth in marinated pork during storage at 15 °C, in the present study, may be attributed to the effects of different meat species and marination sauce composition. The calculated lag phase for each antimicrobial treatment (Baranyi model) is shown in Table 1. During storage at 15 °C, the lag phase in unirradiated samples significantly increased ($P < 0.05$) by 300 h with the addition of 200 ppm Citricidal. Likewise, the lag phase duration of irradiated samples significantly increased ($P < 0.05$) from 520.23 to 633 h. Thus, the lag period significantly increased ($P < 0.05$) in both irradiated and nonirradiated samples supplemented with 200 ppm Citricidal compared to the growth in samples with no Citricidal. Growth at 15 °C was not observed in samples supplemented with 800 ppm Citricidal. Regardless of antimicrobial treatment, lag phase durations obtained in this study were considerably longer than those reported by Juneja and others (1999) in microbiological media (154.8 h).

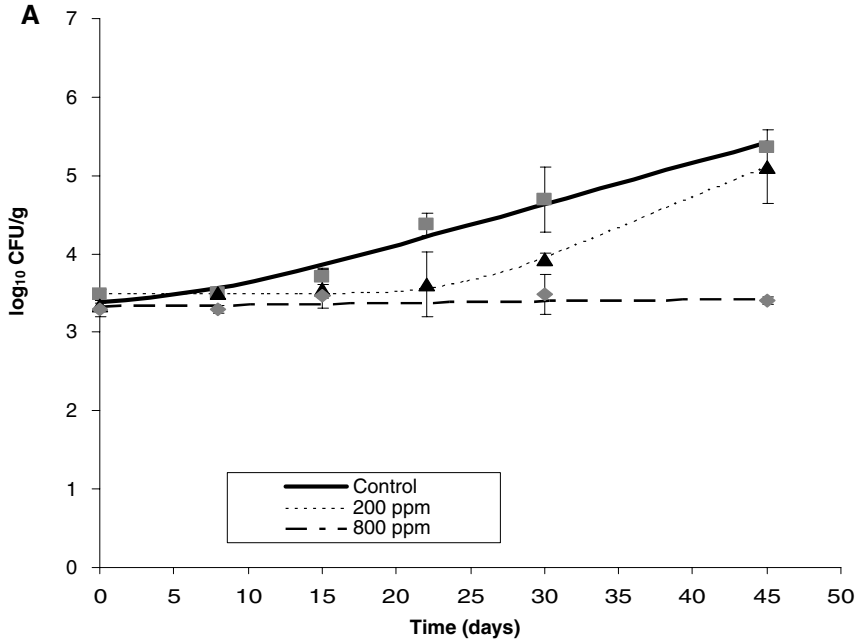


**Figure 1—Microbial counts of *Clostridium perfringens* from spore inocula at 15 °C in *sous-vide* cooked marinated pork meat with prior application of 2 kGy of gamma irradiation (A) or 0 kGy of gamma irradiation (B). All samples were supplemented with 0 (■), 200(▲), and 800(◆) ppm of Citricidal. Nonlinear regressions by Baranyi equation in bold lines are also shown.**

**Table 1—Lag phase duration and regression coefficients ($R^2$) calculated by Baranyi equation of *C. perfringens*[A] in *sous-vide* cooked marinated pork meat from spore inocula supplemented with different levels of Citricidal and with or without prior gamma application, at 15 and 25 °C of storage.**

| | 15 °C Lag time (h) | | | | 25 °C Lag time (h) | | | |
|---|---|---|---|---|---|---|---|---|
| Citricidal (ppm) | 0 kGy | $R^2$ | 2 kGy | $R^2$ | 0 kGy | $R^2$ | 2 kGy | $R^2$ |
| 0 | 357.0 ± 10. 9[a] | 0.98 | 520.5 ± 23.4[d] | 0.98 | 8.8 ± 1.5[a] | 0.94 | 12.7 ± 3.1[a] | 0.93 |
| 200 | 721.1 ± 29.5[b] | 0.97 | 633.0 ± 14.7[b] | 0.99 | 11.8 ± 1.2[a] | 0.91 | 12.9 ± 1.2[a] | 0.92 |
| 800 | ND | ND | ND | ND | 13.2 ± 3.0[a] | 0.90 | 13.9 ± 2.9[a] | 0.94 |

[A]Means ± standard deviation.
Different letters within columns indicate significant difference ($P < 0.05$).
ND = not determined.

Similarly, lower lag phase duration values (200.5 h) were reported in vacuum-packaged cooked beef at 15 °C (122.6 h) and in vacuum-packaged beef with 0.3% of sodium pyrophosphate (Juneja and others 1994; Juneja and Majka 1995).

Figure 2 shows *C. perfringens* growth curves and Baranyi model regressions in marinated pork meat with (1) and without (2) gamma irradiation and different Citricidal levels, subsequently stored at 25 °C. *C. perfringens* population increased to 7.5 $\log_{10}$ CFU/g after 26 h of storage at 25 °C, regardless of the Citricidal concentration or the application of gamma irradiation. It is important to consider that at levels between $10^6$ to $10^8$ cells per gram of product, *C. perfringens* could cause food poisoning symptoms of diarrhea and abdominal pain. Similar results were reported by Juneja and others (2006a) in cooked cured pork meat after 24 h at 26.7 °C. However, in a previous study with Citricidal in marinated *sous vide* chicken stored at 25 °C (Juneja and others 2006b), levels of 7 $\log_{10}$ CFU/g reached within 16 h. It is important to mention that the composition of sauce used in this study was different than the one used in the previous study with chicken (Juneja and others 2006b). Therefore, differences may not be attributed only to meat species. At 25 °C, no significant differences ($P > 0.05$) on the lag phase duration due to antimicrobial treatments (Citricidal and gamma irradiation) were observed (Table 1). The mean lag phase duration was 12.2 h. Using the logistic equation, Juneja and others (1999) calculated a lag phase of 7.1 h when *C. perfringens* was inoculated in microbiological medium (trypticase, peptone, glucose, and yeast extract) incubated at 25 °C. Using the Gompertz equation, Juneja and Majka (1995) reported a lag phase of 12.8 h during storage at 28 °C of vacuum-packaged beef supplemented with 0.3% of pyrophosphate at pH 5.5. Differences in lag phase duration could be attributed to the availability of growth factors in culture media and food products.

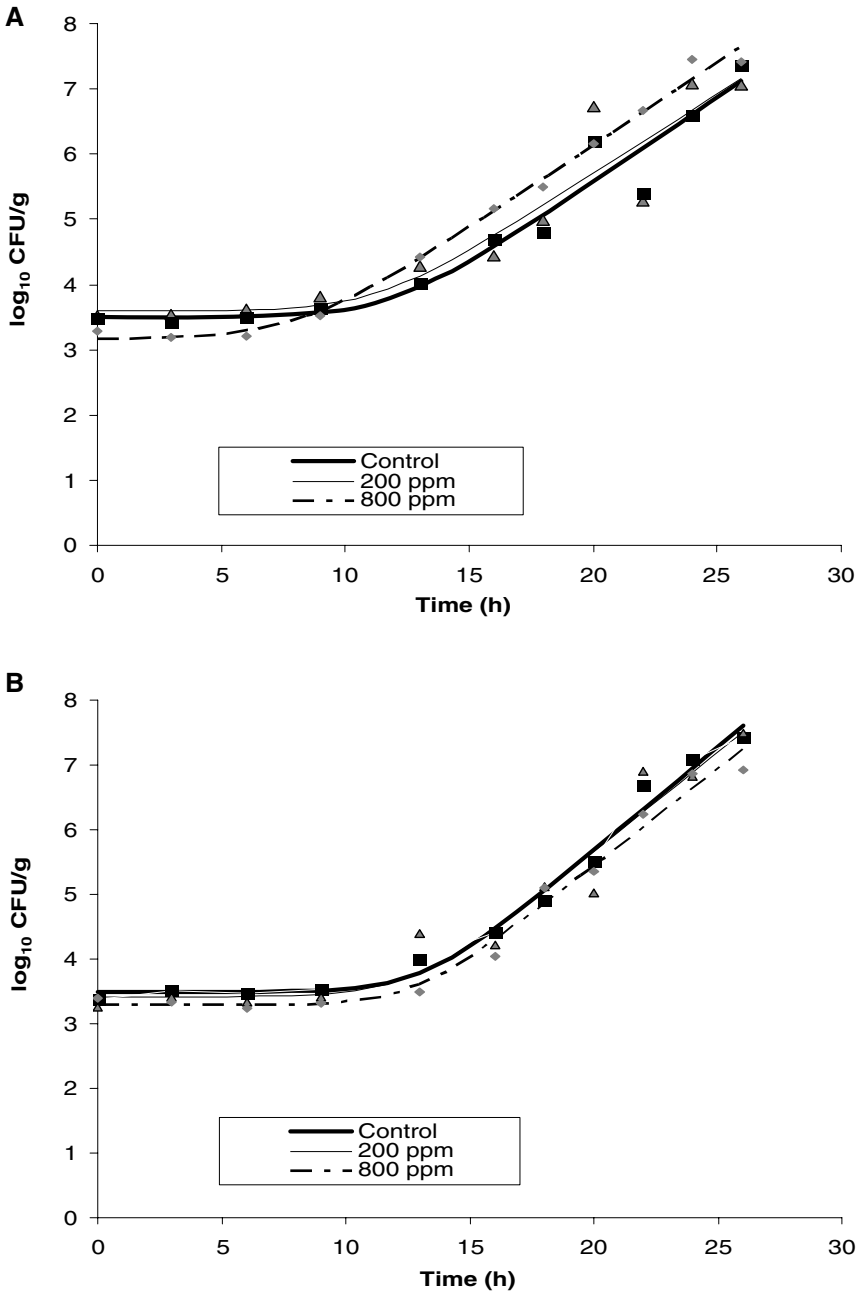


**Figure 2—Microbial counts of *Clostridium perfringens* from spore inocula at 25 °C in *sous-vide* cooked marinated pork meat with prior application of 2 kGy of gamma irradiation (A) or 0 kGy of gamma irradiation (B). All samples were supplemented with 0 (■), 200(▲), and 800(◆) ppm of Citricidal. Nonlinear regressions by Baranyi equation in bold lines are also shown.**

Simulating conditions that may occur during transportation, distribution, storage, or handling in supermarkets or by consumers, the effect of static temperature abuse on *C. perfringens* growth in marinated pork samples was assessed by transferring samples stored at 4 °C on their scheduled sampling day (7, 14, 21, and 28 d of storage) to 25 °C for up to 15 h. There was no significant difference on *C. perfringens* population increase, compared to the control, due to gamma irradiation and supplementation with Citricidal in samples abused for 15 h at 25 °C (data not shown). Thus, temperature abuse of refrigerated products for 15 h would not lead to the growth of *C. perfringens* to high infective dose levels.

No changes were observed in Aw due to the addition of Citricidal or exposure to gamma irradiation. An Aw of 0.97 was maintained during storage at different temperatures ($P > 0.05$). No statistical differences ($P > 0.05$) were found in changes of pH during storage at 25, 15, and 4 °C. A pH of 5.49 was maintained during storage at these conditions.

## Conclusions

Citricidal at 200 ppm inhibited *C. perfringens* spore germination and outgrowth in *sous-vide* Mexican pork entrée at mild temperature abuse conditions of 15 °C. However, growth at 15 °C was completely restricted in the presence of 800 ppm Citricidal. The antimicrobial effect of Citricidal was not observed at 25 °C. Maintenance of good cold chain throughout the distribution is advocated to guard against C. *perfringens* in *sous-vide* processed pork meat marinated with tomatillo sauce. Further studies are recommended on the sensory attributes of the product supplemented with Citricidal.

## References

Baranyi J, Roberts T. 1994. A dynamic approach to predicting bacterial growth in food. Int J Food Microbiol 23:277–94.

Cvetnic Z, Vladimir S. 2005. Antimicrobial activity of grapefruit seed and pulp ethanolic extract. Acta Pharm 54:243–50.

Gombas D, Gomez R. 1978. Sensitization of *Clostridium perfringens* spores to heat by gamma radiation. Appl Environ Microbiol 36:403–7.

Juneja V, Majka W. 1995. Outgrowth of *Clostridium perfringens* spores in cook-in-bag beef products. J Food Safety 15:21–34.

Juneja V, Call J, Miller A. 1993. Evaluation of methylxanthines and related compounds to enhance *Clostridium perfringes* sporulation using a modified Duncan and Strong medium. J Rapid Meth Automation Microbiol 2:203–18.

Juneja V, Marmer B, Miller A. 1994. Growth and sporulation of *Clostridium perfringens* in aerobic and vacuum-packaged cooked beef. J Food Prot 57:393–8.

Juneja V, Whiting R, Marks H, Snyder O. 1999. Predictive model for growth of *Clostridium perfringens* at temperatures applicable to cooling of cooked meat. J Food Microbiol 16:335–49.

Juneja V, Huang L, Thippareddi H. 2006a. Predictive model for growth of *Clostridium perfringens* in cooked cured pork. Int J Food Microbiol 100, 85–92.

Juneja V, Fan X, Peña-Ramos A, Diaz-Cinco M, Pachecho-Aguilar R. 2006b. The effect of grapefruit extract and temperature abuse on growth of *Clostridium perfringens* from spore inocula in marinated, *sous-vide* chicken products. Innova Food Sci Emerg Technol 7:100–6.

Leistner L. 2000. Basic aspects of food preservation by hurdle technology. Int J Food Microbiol 55:181–6.

[NEPA] National Food Processors Association. 1998. Safety considerations for new generation refrigerated foods. Dairy Food Sanit 8:5–7.

Schellekens M. 1996. New research *sous-vide* issues in cooking. Trends Food Sci Technol 7:256–62.

Subba M, Soumithri T, Suryanarayana R. 1967. Antimicrobial action of citrus oils. J Food Sci 32:225–7.